Photobleaching of Red Fluorescence in Oral Biofilms

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Key Words:

Oral biofilm, plaque, fluorescence, quantitative light-induced fluorescence digital (QLFD), porphyrin, photobleaching

Abstract

Background and Objective: Many species of oral bacteria can be induced to fluoresce due to the presence of endogenous porphyrins, a phenomenon that can be utilised to visualise and quantify dental plaque in the laboratory or clinical setting. However; an inevitable consequence of fluorescence is photobleaching, and the effects of this on longitudinal, quantitative analysis of dental plaque has yet to be ascertained.

Material and Methods: Filter membrane biofilms were grown from salivary inocula or single species (*Prevotella nigrescens* and *Prevotella intermedia*). The mature biofilms were then examined in a custom-made lighting rig comprising of 405 nm light emitting diodes capable of delivering 220 W m^{-2} at the sample, an appropriate filter and a digital camera; a set-up analogous to quantitative light-induced fluorescence digital (QLFD). Longitudinal sets of images were captured and processed to assess the degradation in red fluorescence over time.

Results: Photobleaching was observed in all instances. The highest rates of photobleaching were observed immediately after initiation of illumination, specifically during the first minute. Relative rates of photobleaching during the first minute of exposure were; 19.17, 13.72, and 3.43 (arbitrary units per minute) for *P. nigrescens* biofilms, microcosm biofilm and *P. intermedia* respectively.

Conclusion: Photobleaching could be problematic when making quantitative measurements of porphyrin fluorescence *in situ*. Reducing both light levels and exposure time, in combination with increased camera sensitivity, should be the default approach when undertaking analyses by QLFD.

Introduction

Porphyrins and oral bacteria

Fluorescent porphyrins are present in many members of our indigenous microbiota (1, 2), including those found in the oral cavity (3-5). Whilst many bacterial porphyrins are associated with photosynthesis, a relatively large amount of haem (iron protoporphyrin IX) for example is incorporated on the cell surface of the putative periodontal pathogen *Porphyromonas gingivalis* to protect it from hydrogen peroxide (6) with similar processes occurring in *Prevotella nigrescens* and *Prevotella intermedia* (7) (both formerly classified as *Bacteroides melanogenicus*) (8) . The molecular fluorescence of bacterial porphyrins is an adventitious phenomenon which results from the absorption of a photon and the subsequent re-emission of another photon of a longer wavelength as the electrons in the molecule return from the excited (triplet) state to the ground state. Specific porphyrins have distinct excitation spectra with different maxima; protoporphyrin at 593 nm and coproporphyrin 604 nm (9). The wavelengths suitable for the efficient fluorescent excitation of bacterial porphyrins range from near ultraviolet (300 nm) to blue (450 nm). The discrepancy between the colour of the incident light and the fluorescent emission, a phenomenon known as the Stokes shift (10), allows for the selective capture and quantification of the emitted light via an appropriate filter set-up.

Photobleaching occurs when a fluorophore is irreversibly damaged so that it no longer fluoresces. Although the exact mechanisms by which photobleaching occur are not clear, it has been suggested that the fluorophores undergo an oxidative reaction with highly reactive oxygen species such as singlet oxygen ($^1O_2$) and

hydroxyl radicals (OH·) (11). Molecules already in the excited (singlet) state can also be destructively excited by an additional excitation photon, an event dubbed two-photon excitation, but it is unlikely that this process would occur in the experiments discussed herein. Other results have demonstrated photobleaching reactions occurring between excited dye molecules (12) . The generation of highly reactive oxygen species has also been demonstrated to cause cell death in *P. gingivalis*, *P. nigrescens* and *P. intermedia* by the excitation of endogenous porphyrins (13). Lethal photosensitisation (photodynamic therapy) either by the application of photosensitising agents or via endogenous porphyrins has the potential to be an effective means of treating plaque-related diseases (14) .

Fluorescence microscopy techniques can utilise the kinetics of photobleaching by fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP) to reveal rates of diffusion within cell membranes, organelles (15) and biofilms (16). However, when undertaking quantitative measurements of fluorescence, photobleaching can be problematic (17).

Quantitative light-induced fluorescence

Quantitative Light-induced Fluorescence (QLF) uses violet light to induce fluorescence in tooth enamel and collects the resulting emissions via a high band-pass filter (>520 nm) in conjunction with a computer-controlled digital camera (18). When viewed under QLF lighting conditions, areas of demineralised enamel fluoresce less than surrounding sound enamel and so appear darker. Regions of

demineralised enamel are visible under QLF lighting conditions before they are visible to the eye as white spot lesions (19). Although QLF was initially developed for the analysis of tooth enamel (20), it has been subsequently demonstrated to be capable of revealing dental plaque due to the fluorescence of endogenous porphyrins (5). However, since the filter configuration of QLF was designed to maximise the transmission of green light there are limitations as to its usefulness for the analysis of dental plaque (21). Quantitative Light-Induced Fluorescence Digital (QLFD) is an adaptation of QLF which employs a modified filter set (D007, Inspektor Research Systems BV, Amsterdam, Netherlands), narrow-band violet light (405 nm) and a high-specification digital SLR camera. This configuration has been specifically developed to enhance the visualisation and quantification of plaque. During clinical investigations to assess plaque, QLFD is typically used to identify regions of red fluorescence and capture a sequence of images at different visits in order to quantify the progression of conservative dental treatment.

A better understanding of photobleaching phenomena with respect to indigenous bacterial porphyrins, illuminated by QLFD lighting, *in situ* is required to enable accurate quantitative analyses of dental plaque to be undertaken.

Materials and Methods

Filter membrane biofilms

Approximately 10 ml of unstimulated saliva was obtained from a healthy volunteer with no previous history of periodontitis. This was split into 1 ml aliquots and frozen. Nitrocellulose filter-membranes (47 mm diameter, 0.45 µm pore size, Invitrogen Ltd.,

Paisley, Renfrewshire, UK) were laid, with their inked grid upwards, on top of blood agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood. A 50 µl aliquot of the saliva sample was spread over the membrane before being incubated at 37ºC in anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for seven days to allow microcosm oral biofilms to develop. Individual biofilm laden filter membranes were removed from the supporting agar and placed in a Petri dish which had first had 200 µl of phosphate buffered saline (PBS) beaded over the surface to help prevent the membrane biofilm from drying out. Similar single-species biofilms were grown using heavy colony inocula of *Prevotella nigrescens* (ATCC 25261) (seven day old cultures) or *Prevotella intermedia* (ATCC 25611) (five day old cultures) suspended in 1 ml of PBS.

Fluorescence imaging

A custom-made rig, incorporating QLFD technology, was constructed to enable the capture of fluorescent images under reproducible lighting conditions from surface-mounted indium gallium nitride light emitting diodes (LED) (EWC 400 SC2C, radiant power 600 mW, 23º beam angle; E Wave Corporation, London, UK) with a wavelength band from 400 nm to a peak output at 405 nm (violet). To construct the QLFD *in vitro* rig, an LED was soldered onto the outside of a copper ring, being a section of standard domestic plumbing material, which acted as a heat-sink to prevent overheating. Three such mounted LEDs were then fixed inside an approximately hemispherical plastic bowl so that the light beams converged on the sample (Figure 1). A hole was cut into the base for the unimpeded viewing of the sample by a camera, with another hole in the sidewall to allow the sample to be

136 easily manipulated. The LEDs were powered by a DC adaptor with an output of 5
137 volts at 1.2 amps connected in parallel. The distance between the LEDs and the
138 sample was 100 mm at an angle of incidence between camera and LEDs of 30º from
139 the surface normal. The light incident on the sample was measured as irradiance by
140 a photosynthetically active radiometer (PAR) with a cosine corrected detector (Q201
141 PAR with SD221Q Cos detector, Macam Photometrics Limited, Livingston, UK). A
142 cut-off filter (D007, Inspektor Research Systems BV, Amsterdam, Netherlands) was
143 placed in front of the camera lens to minimise the transmission of light close to the
144 excitation wavelength whilst maximising the transmission of the red part of the
145 spectrum. All illumination / photobleaching experiments were undertaken in a dark-
146 room.

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148 Images were captured with a 'live view' enabled digital SLR camera, (Model: 1000D,
149 Canon, Tokyo, Japan) equipped with a 60 mm, *f*/2.8 macro lens (Model: EF-S,
150 Canon) connected to a computer. Proprietary software (C2 v1.0.0.7, Inspektor
151 Research Systems BV) was used to control the camera and store the images. Low
152 apertures (i.e. *f*/2.8 to *f*/8) and ISO settings of 200 – 400 were typically used to
153 maximise the light sensitivity of the camera without adversely affecting image quality.
154 The camera's on-board 'custom white balance' feature was calibrated against a
155 sheet of white paper before fluorescence imaging to effectively eliminate the
156 colouration of the filter in the resulting images. The camera resolution was set to
157 'low' (3.4 megapixels) to facilitate the processing of large numbers of data files in the
158 form of uncompressed 24-bit bitmap files. The LEDs were switched on for at least
159 10 minutes prior to use to allow their temperature to stabilise. Without moving the
160 sample, or changing camera settings, a series of images was captured over time

using the in-built image sequencer incorporated into the control software. Control experiments included membranes that were partially covered with aluminium foil to shield portions of them from the light. Four separate microcosm biofilm photobleaching experiments were undertaken whilst the experiments for single species were conducted in duplicate.

Image analysis

Images were analysed with an open source software package (ImageJ 1.43q, The National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). The images comprising the time-lapse sequence were opened with ImageJ and compiled into a single image 'stack'. The stack was then split into its red, green and blue (RGB) component colour channels; to isolate the red channel as an 8-bit greyscale (i.e. pixel brightness values from 0 to 255). A user-defined 'rectangular selection' region of interest (ROI) was created within one of the inked grid squares on the filter membrane. The size of the grid squares was 4 mm x 4 mm and the ROI encompassed approximately 20 000 pixels. The 'z-axis profile' of the ROI was then measured through the image stack and the mean pixel brightness values at each time point were copied into Microsoft Excel. The ROI was then moved to an adjacent square on the grid and the process repeated to give a total of eight discrete counts from the same biofilm sample. The mean pixel brightness values from the eight sample sights from the were then themselves averaged before being normalised to 100 at time zero to yield the arbitrary unit used throughout these experiments; 'normalised mean pixel brightness' (NMPB), which allowed the direct comparisons to be made between separate experiments.

Changes in fluorescence (ΔF) were calculated in terms of the shift in NMPB per unit time to yield results in terms of ΔF per minute. These ΔF values were then allocated into sub-sets to determine mean ΔF between discrete time points within the photobleaching experiment; from 0 to 1 minute, 1 to 2 minutes, 2 to 5 minutes, 5 to 10 minutes and 10 to 20 minutes.

Inter-operator reliability

Two researchers (CKH and MRTF) independently analysed the image stacks from two photobleaching experiments in order to ascertain the reliability of the methods previously described. This exercise was undertaken in light of the possible variability due to manual selection of the ROI parameters; namely: size of ROI, placement of ROI within the membrane square and choice of the eight membrane squares used for analysis. Results were tested using Pearson Correlation with PASW Statistics 17.0 (equivalent to SPSS) (Polar Engineering and Statistics).

Results

Light exposure

The configuration of the LEDs at an angle of incidence to the sample of 30º (Figure 1) corresponded to a radiant intensity of 0.87 per unit solid angle (Lambert's cosine law). The angle of incidence was within the angular response parameters of the cosine corrected PAR detector. The maximum radiometer reading at the sample

location was 750 µmol m^{-2} s^{-1}, which is equivalent to 220 W m^{-2} at 405 nm. Light leakage from the LEDs was minimal, being measured at 1.8 µmol m^{-2} s^{-1} immediately outside the obvious pool of light from a single LED. Due to the build-up of heat during operation, the LED's light output dropped to 89% of their initial power after 10 minutes usage after which time their output stabilised (data not shown).

Biofilm photobleaching

Photobleaching was evident in all of the samples analysed in this study; microcosm oral biofilm, *P. nigrescens* and *P. intermedia* single-species biofilms. A control experiment was designed to confirm that light was responsible for the reduction in fluorescence in which half of the sample was shielded from direct illumination with aluminium foil. After 25 minutes, the NMPB on the exposed half dropped to 31.23 whilst the covered half was 84.73 (figure 2).

Figure 3 shows the results from an individual QLFD experiment which demonstrates photobleaching as a decrease in NMPB over time. NMPB data such as these were collated to yield means along with their corresponding standard deviations (Figure 4).

Rates of photobleaching, expressed as ΔF were highest during the initial stages of exposure to light (i.e. the first minute). In the case of microcosm biofilms and *P. nigrescens* biofilms, photobleaching rates fell to approximately half their initial value after 10 minutes exposure (Figure 5). Initial rates of photobleaching during the first

minute of exposure, expressed as ΔF (arbitrary units normalised to 100 at time zero) per minute, were; 19.17, 13.72, and 3.43 for *P. nigrescens* biofilms, microcosm biofilm and *P. intermedia* respectively. Photobleaching dynamics were reproducible between replicate samples; Pearson correlation coefficient values between the four microcosm biofilm samples ranged from 0.993 to 0.997 and were significant at the 0.01 level (2-tailed).

Inter-operator reliability

Pearson correlation coefficients for the two photobleaching experiments subjected to inter-operator reliability testing were significant at the 0.01 level (2-tailed) with correlations of 0.992 and 1. The data presented herein was the first set of data that was analysed.

Discussion

Light exposure

A PAR detector was chosen to measure the light irradiance from the custom rig since photobleaching is dependent on the amount of light energy incident per unit surface area and not the power output of the LEDs *per se* (22). The camera / image analysis method of quantifying pixel brightness proved far more sensitive to subtle changes in ambient lighting conditions than the results from the PAR detector would have suggested. The data collected from the automated image sequencer yielded fewer perturbations in the fluorescence curve than when captured manually during preliminary experiments. This suggested that variation in lighting from the laptop

screen, reflected off the white laboratory coat worn by the operator, was detectable in the analysed images. The inter-operator reliability data suggests that the image analysis methods employed were robust and reproducible.

Whilst every effort was made to focus the light onto the centre of the filter membrane, an *ad-hoc* observation made using the PAR revealed that a single representative attempt to place the detector in the 'bright centre of the light beams' by the unaided eye, yielded only 73% of the actual maximum irradiance obtainable by scrutinising the meter readings. The difference between these two positions was of the order of 10 mm. Although the foci of the LEDs were generally convergent onto the sample, it is unclear how the extent of photobleaching relates to specific positions within the pool of light incident on the sample. For example, an area with less light incident upon it, will fluoresce less and will likewise have a lower rate of photobleaching (23). The net result of this would be differential rates of photobleaching across a (large) sample and a hypothetical example of this effect is demonstrated in Figure 6. Performing image analysis on adjacent sites on the biofilm membrane will help to minimise the effects of heterogeneous lighting. Another confounding factor that should be considered is the photo-shielding effect (24) which occurs when a relatively high concentration of fluorophore absorbs excitation photons, which in turn reduces the number of photons able to penetrate into deeper layers of the sample. 'Iron porphyrin' can account for up to 50% of the dry weight of the biomass of *Bacteroides* (many of which have been reclassified as *Prevotella spp*.) when growing on blood agar (25) . Photo-shielding could reduce the observed effects of photobleaching due to decreased excitation within the sample as a whole; in other words, there may not be a direct relationship between fluorescence

intensity / photobleaching and net porphyrin concentration within a heterogeneous, three-dimensional microbial biofilm.

Biofilm photobleaching

Rates of photobleaching decreased during exposure to QLFD light for 20 minutes, after which time there was very little further reduction in observed fluorescence. It is unlikely that imaging (exposure) times beyond this would be representative of image capture *in vivo*. A reduction in mean pixel brightness of ~14% after one minute's illumination with QLFD represents an unacceptable inaccuracy for the quantitative analysis of the red fluorescence of dental plaque. Casual viewing and manipulation of a sample / patient under the 405 nm lighting in order to correct the focus, determine other camera settings and image capture will inevitably cause photobleaching. In order to minimise this effect, samples should be positioned and focussed under normal, white-light conditions. It is however an unavoidable fact that in order to observe fluorescence, one must perturb fluorescence.

The rates of ΔF observed suggest that this effect was immediate and replicated the photobleaching kinetics of protoporphyrin IX previously observed in PLC hepatoma cells at 405 nm (26). In the current study, ΔF values were grouped to yield average values for all data points within discrete time bands (0 to 1 minutes, 1 to 2 minutes, 2 to 5 minutes, 5 to 10 minutes and 10 to 20 minutes) to obviate the confounding effects of individual data points with a positive ΔF value amongst predominantly negative ΔF (i.e. fluorescence decreasing) values. During a longitudinal study, a

system whereby the LEDs are only illuminated during imaging should be employed. This will also maximise the power output of the LEDs as they emit more light when they are at ambient (room) temperature as opposed to once they have warmed to their operating temperature. Using the PAR detector it was determined that the irradiance supplied by the lighting rig did not decrease due to heating effects when operated for 5 seconds out of every minute, similarly lighting for 5 seconds out of every 30 seconds only reduced light output to 99.45% (data not shown).

The differential fluorescence of oral anaerobic bacteria under ultraviolet light has been suggested as a tool for their rapid identification. The fluorescence previously observed in strains of *Bacteridoes* (now reclassified as *Prevotella spp*. and *P. gingivalis*) encompasses a colour range that has been described as; red, yellow, red-orange, brilliant red, pink-orange, orange, yellow-orange, and red-brown (27) . These colours also changed with age of the culture and are almost certainly a manifestation of the sequential metabolism of porphyrins (28) . No fluorescence was observed in *P. gingivalis* at any time, including when emulsified in methanol – a technique which can reveal fluorescence in older cultures which have lost this capacity (2) . The inability of *P. gingivalis* to fluoresce is probably due to the deposition of haem as an µ-oxo dimer on the cell surface, as opposed to the monomeric form in *Prevotella* (6, 7). It was observed in preliminary experiments that the fluorescence of *P. intermedia* was greatly diminished when incubated for seven days, hence the use of younger (five day) cultures.

An additional experiment was conducted to see if it was possible to use a biofilm-laden filter membrane as a rudimentary photographic plate to create a recognisable image. An acetate mask depicting the University of Liverpool logo was placed in front of a microcosm biofilm and exposed to QLFD light. After 16 minutes, the mask was removed to reveal a photobleached logo on the membrane (see supplementary information).

Fluorescence imaging has the potential to be a useful tool for quantifying dental plaque in the research environment, both in terms of the amount of fluorescent material and the 'quality' of its fluorescent properties in terms of red and green fluorescence (29). The methodology described herein for measuring photobleaching of red fluorescence in microbial biofilms appears to be robust and reproducible. However; the destruction of the endogenous fluorophores within dental plaque by photobleaching phenomena, when viewed with QLFD or similar technologies, needs to be considered and steps taken to curtail this effect in both the research and clinical environments such as improving camera sensitivity (i.e. low shutter speeds, low apertures and high ISO settings), filter characteristics and keeping irradiance to a minimum. The reported studies do not imply that this technique has clinical applicability, but provides proof of principle data. Issues such as biofilm thickness and degree of pigmentation may influence the penetration of both the excitation and emitted fluorescent light and therefore the rate of photobleaching *in vivo*. Further research is required to determine the effects of photobleaching on plaque fluorescence.

Acknowledgments

This work was undertaken as a final year research project by MRTF and was funded internally by the University of Liverpool, School of Dental Sciences and Department of Human Anatomy and Cell Biology.

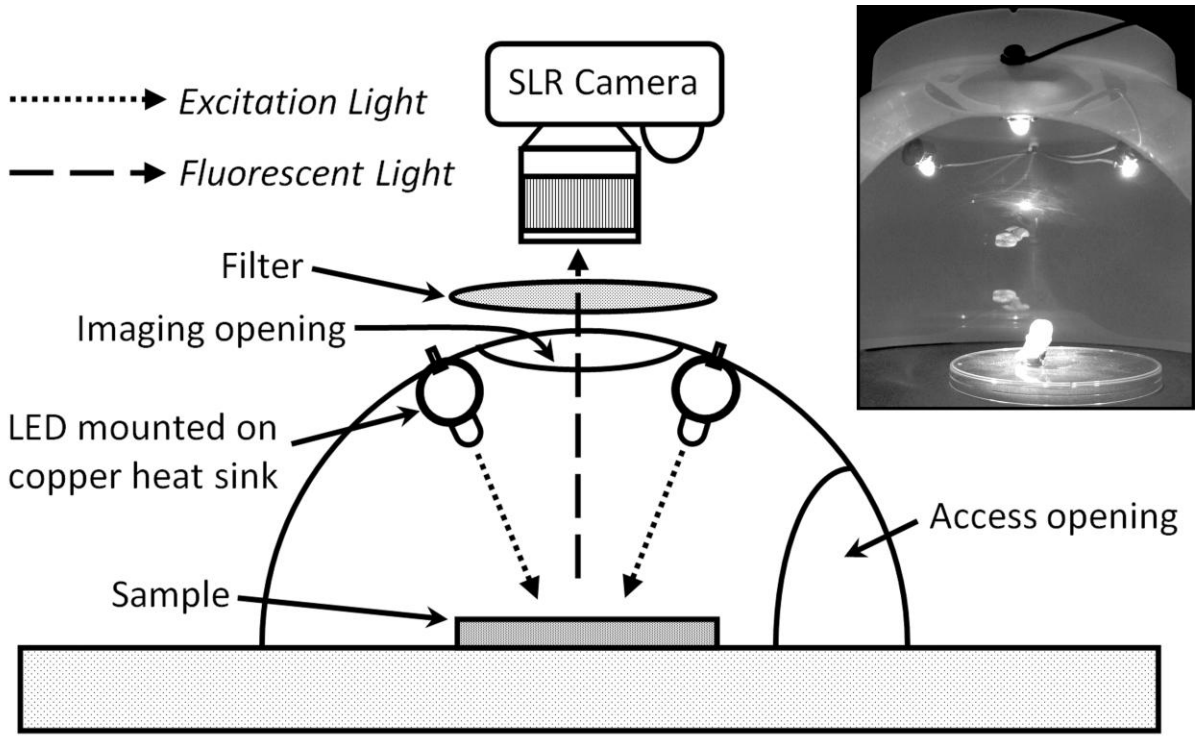


Figure 1. Schematic diagram of the 405 nm lighting rig, comprising of three equidistant indium gallium nitride light emitting diodes (inset: a tooth sample illuminated by the lighting rig).

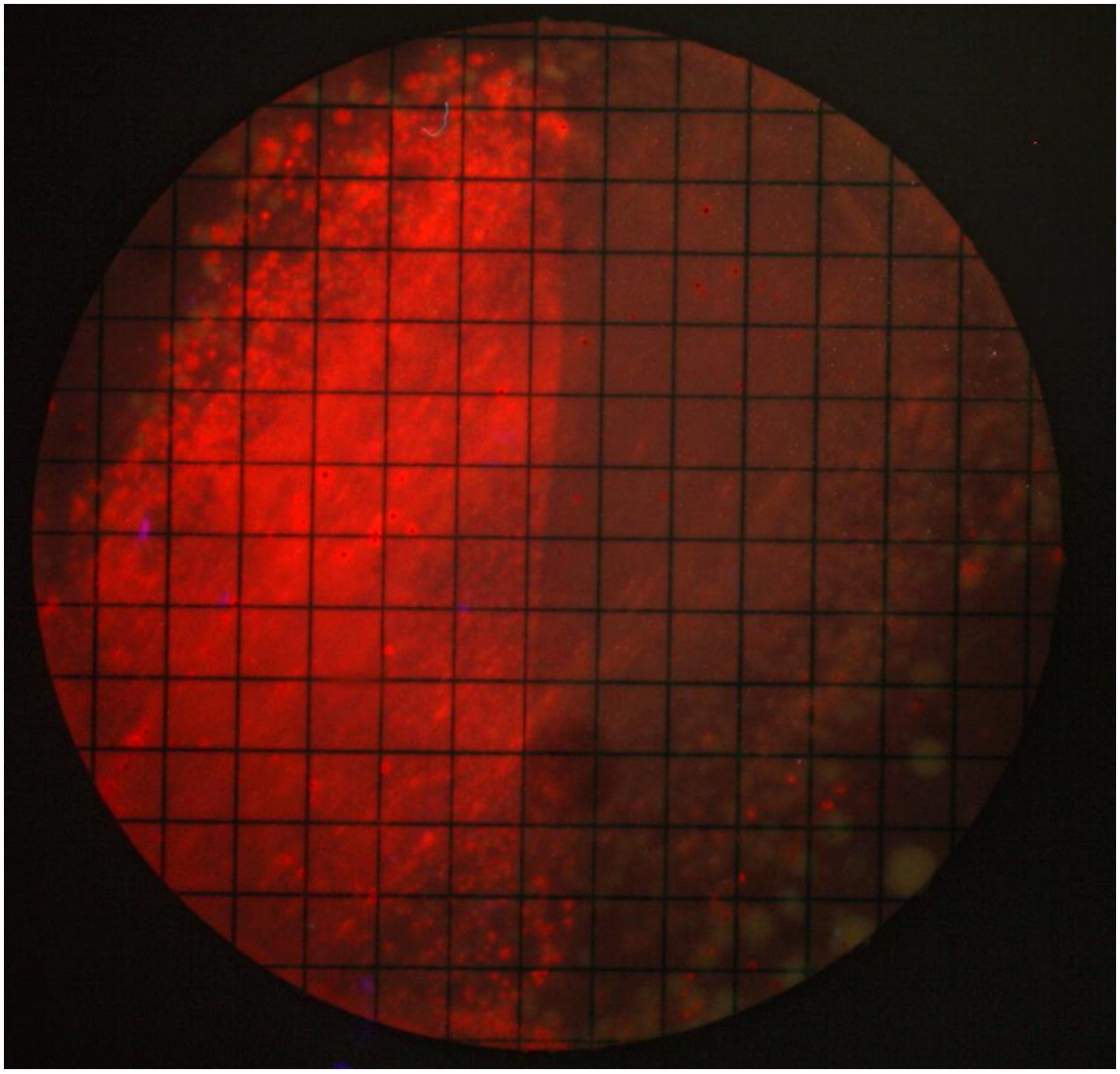
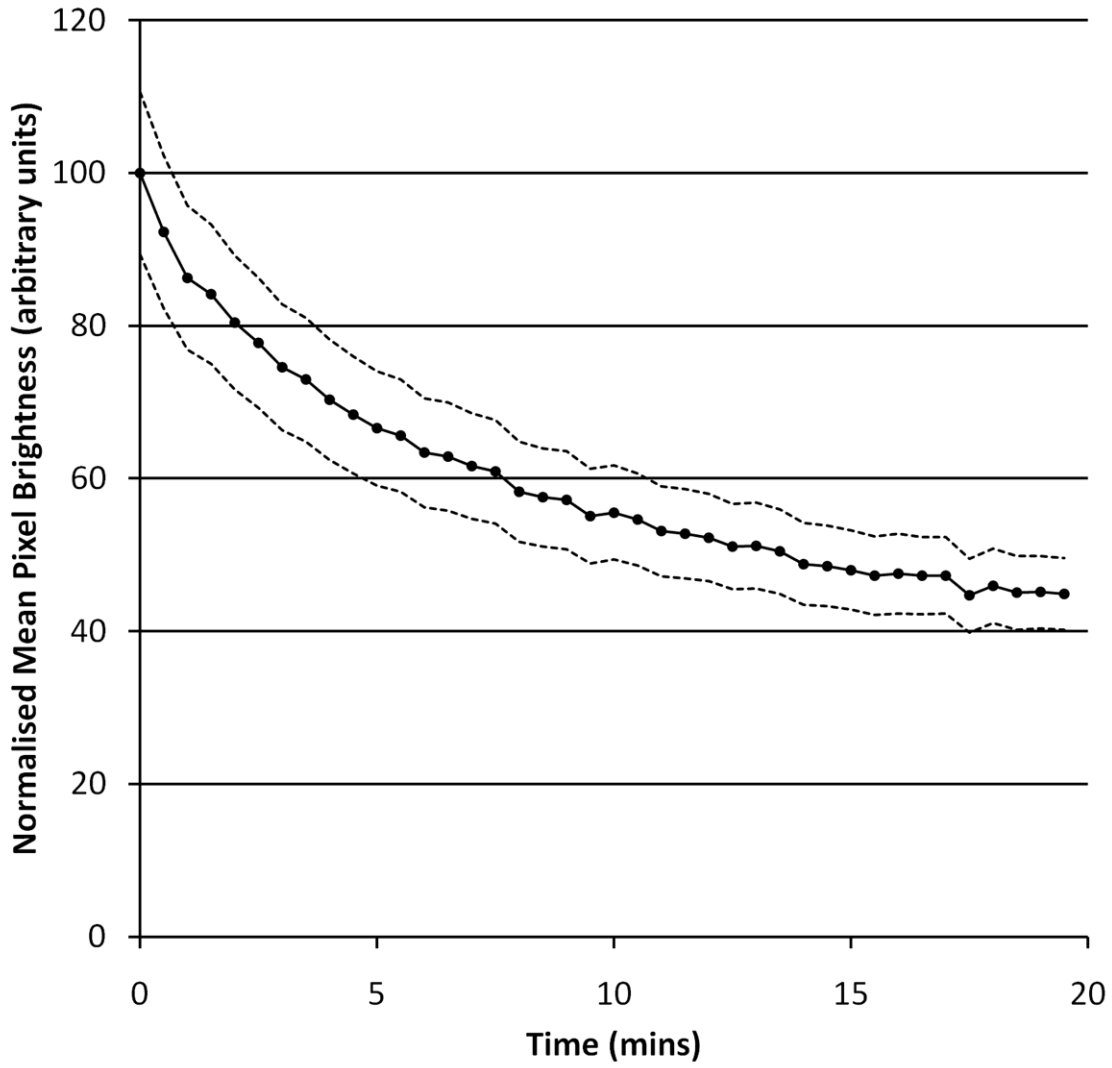


Figure 2. Microcosm filter-membrane biofilm viewed under the QLFD lighting system. Photobleaching was demonstrated on the right-hand side of the membrane in this instance by previously covering the left-hand side of the membrane with aluminium foil whilst 405 nm light at 750 µmol m^{-2} s^{-1} (maximum) was incident onto the sample for 25 minutes. The foil was removed immediately before this image was captured.



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369 Figure 3. A representative microcosm biofilm photobleaching experiment following
370 illumination with the QLFD lighting system. The solid line is the mean pixel
371 brightness from adjacent regions of interest within the sample (n=8); the dotted lines
372 show standard deviations.

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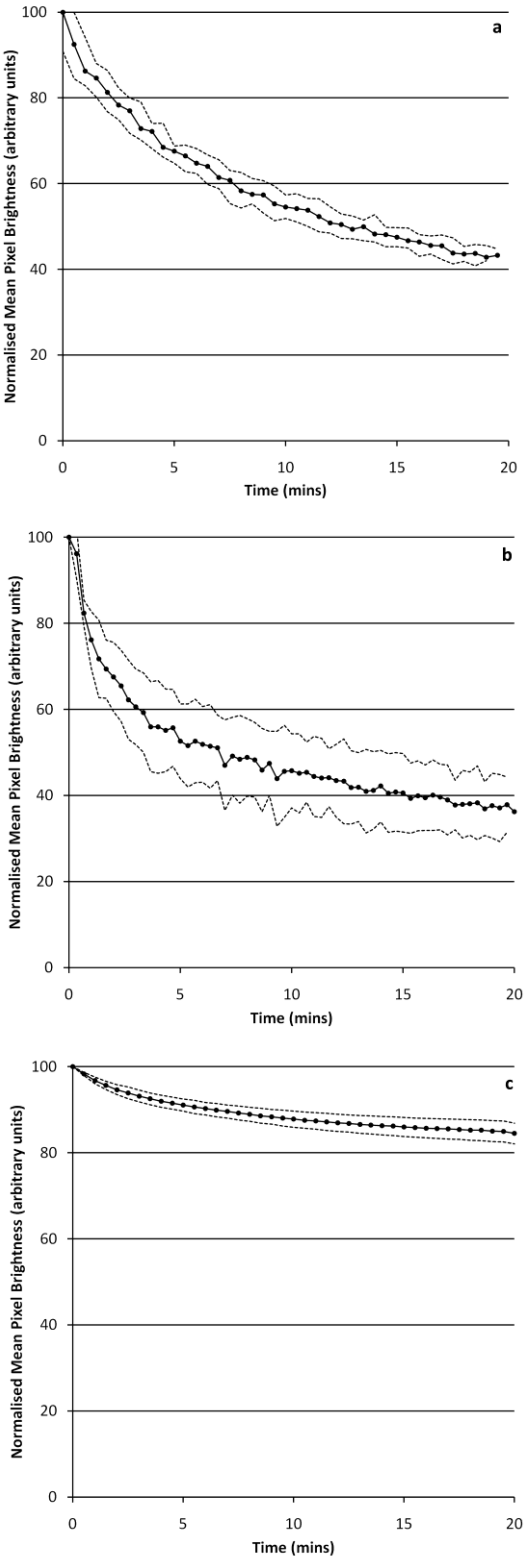

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376 Figure 4. Mean red fluorescence observed in microcosm (n=4) (a), *P. nigrescens*
377 (n=2) (b) and *P. intermedia* (n=2) (c) filter-membrane biofilms viewed under QLFD
378 lighting. The solid lines represent the mean values with the dotted lines showing
379 standard deviations.

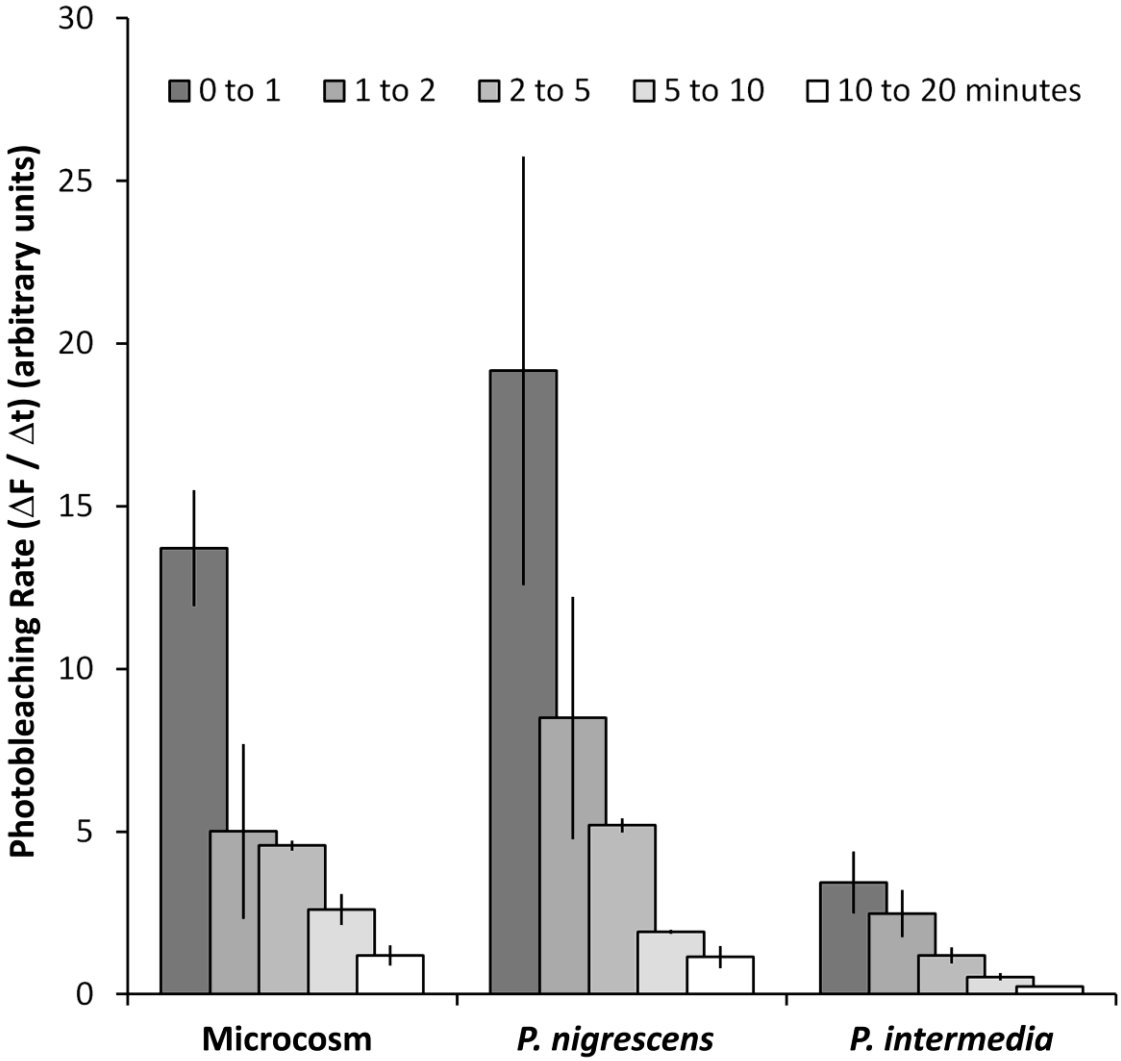


Figure 5. Rates of photobleaching shown as the decrease in fluorescence (ΔF) with time (Δt) within a range of time points; 0 to 1, 1 to 2, 2 to 5, 5 to 10 and 10 to 20 minutes. These data corresponds to Figure 4. Error bars indicate standard deviations.

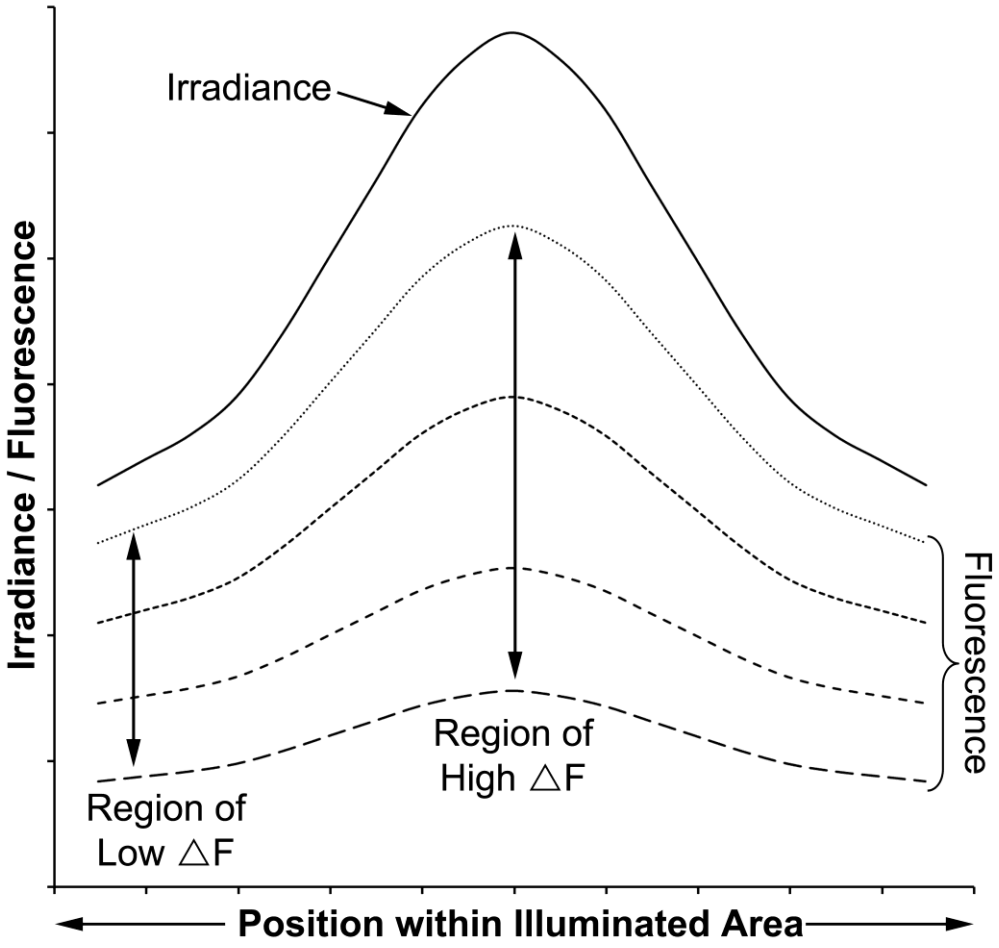
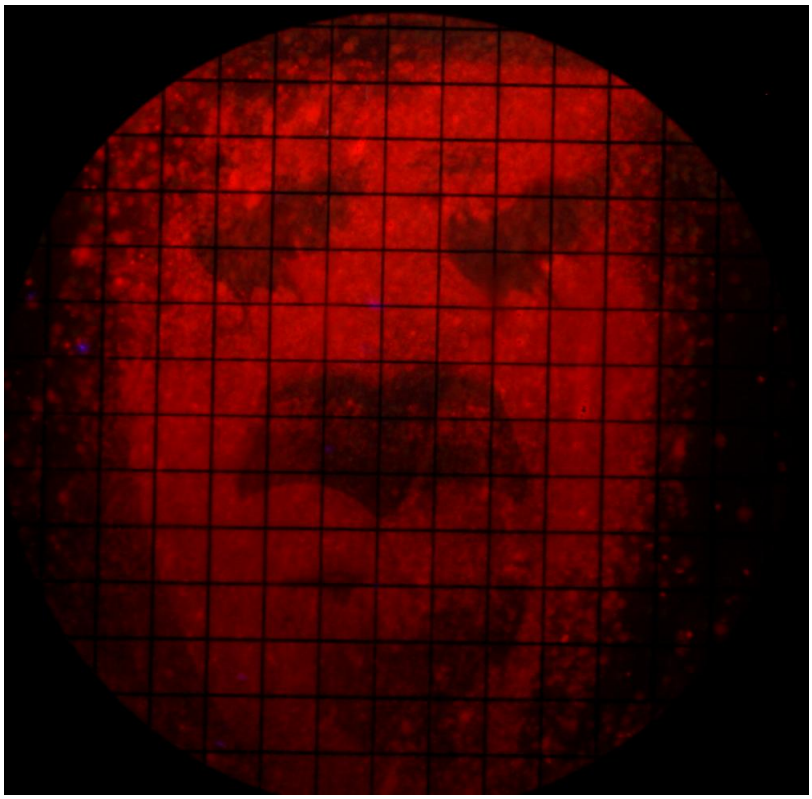


Figure 6. A theoretical model showing differential rates of photobleaching across a sample illuminated with the custom lighting rig. Heterogenous irradiance of the sample is represented by the solid line, whereas the resulting fluorescence values are shown by the dotted lines at four times points. Fluorescence decreases over time and the rate of photobleaching is directly proportional to the incident irradiation.

Supplementary Images



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